EPA+DHA Oral Therapy Reduces High Levels of Circulating Pro-Inflammatory Cytokines in Older Adults with Chronic Wounds

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By

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Abstract

High levels of circulating pro-inflammatory cytokines contribute to inflammaging, the age-related chronic systemic inflammation involved in the pathobiology of numerous chronic conditions common to older adults (e.g., cardiovascular disease (CVD), arthritis, chronic wounds). Low-risk therapies to target the underlying factors linked to inflammaging are critically needed because by 2060 the U. S. population aged 65 years and older is projected to reach 98 million. Some studies have shown that the bioactive elements of fish oil, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), reduce pro-inflammatory cytokine synthesis, but the effects of supplementing diets with EPA+DHA in older adults remain unclear. The purpose of this study was to compare pro-inflammatory cytokine levels over time between older adults receiving EPA+DHA supplementation versus placebo. This randomized, double-blind study evaluated 35 older adults with chronic wounds at a university research center. For 8 weeks, EPA+DHA Group participants (n=16) consumed EPA+DHA supplements (2. 5 g/d) and Placebo Group participants (n=19) consumed a placebo. Fasting blood plasma samples were collected at Weeks 0, 4 and 8 to quantify levels of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α. Sociodemographic, comorbidity, and body mass index (BMI) data were also collected. On average, participants were 60.6 years (*SD*=11. 96) with a BMI of 41. 7 (*SD*=11. 51). The majority were male (60%), Caucasian (74%) and had diagnoses of CVD (77%) and/or arthritis (51%) in addition to chronic wounds. There were no significant differences in age, BMI, or number of comorbidities between the two groups. After adjusting for baseline differences, the EPA+DHA Group had significantly lower levels of IL-6 ($p = .008$), IL-1β ($p < .001$), and TNF-α ($p < .001$) than the Placebo Group at Week 4 and at Week 8 [IL-6 ($p = .007$), IL-1β ($p < .001$), and TNF-α ($p < .001$)]. The findings suggest that low-risk EPA+DHA oral therapy may be

effective for reducing the high levels of circulating pro-inflammatory cytokines linked to inflammaging in older adults. Future studies could test EPA+DHA oral therapy in larger, more diverse samples of older adults.

Chapter I: Statement of the Problem

Introduction

Inflammaging is a term used to describe the chronic, low-grade systemic inflammation that occurs with aging (Franceschi & Campisi, 2014). Inflammaging is characterized by high levels of pro-inflammatory cytokines in venous circulation that are involved in the pathobiology of multiple age-related conditions including cardiovascular disease (CVD), diabetes, arthritis, frailty, and chronic wounds. Inflammaging and the associated high levels of circulating pro-inflammatory cytokines are now considered to be significant risk factors for increased morbidity and mortality in the aging population (Calder et al., 2017; Franceschi & Campisi, 2014). Given that by 2050 the population of adults in the U.S. aged 65 and older is projected to be 83.7 million, low-risk therapies to reduce the harmful effects of inflammaging are greatly needed (Ortman, Velkoff, & Hogan, 2014). The omega-3 (n-3) polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) found almost exclusively in fish oil have anti-inflammatory actions. Moreover, some studies have shown that EPA+DHA can actually reduce the synthesis of pro-inflammatory cytokines by cells involved in inflammation regulation, however the effects of EPA+DHA oral therapy on these inflammatory mediators in older adults have not yet been determined. Thus the purpose of this study was to compare levels of circulating pro-inflammatory cytokines in older adults with at least one chronic inflammatory condition (chronic wounds) receiving either EPA+DHA oral therapy or placebo oral therapy.

Background

In healthy immune systems inflammation is an acute, self-limiting process that is down-regulated by cellular mechanisms such as the release of anti-inflammatory cytokines and the inhibition of inflammatory cell signaling cascades that collectively promote tissue repair (Calder

et al., 2013). With aging, the immune system becomes less responsive and thus less efficient at regulating inflammation. As a result, inflammation can become chronic. Inflammaging, a term used to describe the chronic, low-grade inflammation that occurs with aging, is characterized by high levels of circulating pro-inflammatory cytokines, specifically IL-1β, IL-6, and TNF-α (Franceschi & Campisi, 2014; Minciullo et al., 2015). Ultimately, inflammaging increases risk for many inflammation-related conditions such as CVD, diabetes and chronic wounds that cause considerable morbidity and mortality for older adults (Calder et al., 2017; Franceschi & Campisi, 2014).

The n-3 PUFAs EPA+DHA found in fish oil are metabolized to lipid mediators with strong anti-inflammatory actions (Calder, 2015). Conversely, the n-6 PUFAs (i.e. arachidonic acid [AA]) are metabolized to lipid mediators with primarily pro-inflammatory actions (Wall, Ross, Fitzgerald, & Stanton, 2010). Because the n-3 and n-6 PUFAs are competitively metabolized, the amounts consumed through the diet impact the ratios of pro- to anti-inflammatory mediators generated via PUFA metabolism (Figure 1). Unfortunately, the dietary intake of n-3 PUFAs (including EPA+DHA) in the U.S. is considerably lower than recommended (90 mg/d vs. 250 - 500 mg/d) (Blasbalg, Hibbeln, Ramsden, Majchrzak, & Rawlings, 2011; Flock, Harris, & Kris-Etherton, 2013). Moreover, it has been estimated that Western diets currently contain high n-6:n-3 PUFA ratios in the range of 15:1-16:1 rather than the recommended optimal ratio of approximately 4:1 (Wall et al., 2010). Therefore it is important that older adults consume adequate daily amounts of n-3 PUFAs, particularly EPA+DHA, to help balance the high n-6:n-3 PUFA ratios common in Western diets. By increasing the intake of EPA+DHA, the harmful effects of inflammaging may be reduced.

Purpose of the Study

The purpose of this randomized, double-blind, controlled study was to compare plasma levels of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α within and between two groups of older adults with chronic venous leg ulcers (CVLUs) at 0, 4, and 8 weeks over an 8-week interval. The EPA+DHA group consumed EPA+DHA supplements for 8 weeks and the control group consumed a placebo for the same interval.

Significance of the Study

The chronic diseases associated with inflammaging affect millions of Americans every year, reducing quality of life and increasing the economic burden due to the high healthcare costs required for treatments and hospitalizations (Centers for Disease Control and Prevention, 2017a). Given the projected growth of the aging population, low risk, low cost therapies are needed to address the problem of inflammaging. EPA+DHA therapy has potential to reduce the harmful effects of inflammaging by targeting the high systemic levels of pro-inflammatory cytokines that are linked to inflammaging.

Conceptual Frame of Reference

The theoretical framework used to guide the study design is based on Dr. Philip Calder's theory that EPA+DHA reduce inflammation systemically and locally by: 1) generating lipid mediators that are weak chemoattractants for pro-inflammatory cytokines, and 2) down-regulating the gene expression of pro-inflammatory cytokines by cells involved in inflammation regulation (e.g., neutrophils) (Figure 1) (Calder, 2015).

Study Aims:

Between two groups of older adults (≥ 50 years of age) with CVLUs receiving standard wound care at an OSU outpatient wound clinic and 1) EPA+DHA therapy (2.2g/d of EPA+ 0.6g/d of DHA) for 8 weeks or 2) placebo therapy for 8 weeks:

Aim 1: Compare plasma levels of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α at 0, 4 and 8 weeks.

Aim 2: Compare plasma levels of n-3 and n-6 PUFAs at 0, 4 and 8 weeks.

Chapter II: Review of Literature

Aging is associated with chronic, systemic inflammation, and this phenomenon has been termed "inflammaging" (Franceschi & Campisi, 2014). Inflammaging is involved in the pathobiology of numerous chronic diseases commonly seen in aging (Franceschi & Campisi, 2014). Although the cause of inflammaging is not entirely known, multiple studies have reported that high levels of circulating pro-inflammatory cytokines is a hallmark sign of inflammaging. Given the rising numbers of older adults worldwide, there is a critical need for effective low-risk, low-cost interventions to address the problem of inflammaging. Some studies have shown that the bioactive elements of fish oil, EPA+DHA, safely and effectively reduce levels of some pro-inflammatory cytokines in cell and animal models of inflammation, but their effects in aging adults is not completely known (Calder, 2015).

Inflammaging and Chronic Disorders in Aging

Given that "aging is accompanied by immune, hormonal, and adipose changes leading to a chronic inflammatory state," as the American population ages, the incidence of chronic inflammation and chronic disease will increase (Michaud et al., 2013). Moreover, it has been estimated that nearly 40 percent of adults in the U.S., the majority being older adults, suffer from multi-morbidity, the co-occurrence of chronic disease (Stepanova, Rodriguez, Birerdinc, & Baranova, 2015). The evidence continues to grow that inflammaging contributes significantly to the development and exacerbation of many age-related chronic diseases/conditions such as CVD,

diabetes, and chronic wounds (Cevenini, Monti, & Franceschi, 2013; Makrantonaki, Wlaschek, & Scharffetter-Kochanek, 2017).

CVD remains the leading cause of death for both men and women in the U.S., killing more than 630,000 people each year and costing the healthcare system over $200 billion annually (Centers for Disease Control and Prevention, 2017b). Studies have shown that chronic low grade systemic inflammation plays as much a role in the development of atherosclerosis as increased levels of triglycerides (Christodoulidis, Vittorio, Fudim, Lerakis, & Kosmas, 2014; Golia et al., 2014; Moss & Ramji, 2016). As various lipoproteins accumulate in the vessels, an inflammatory response is triggered. Subsequently, the endothelial lining of the vessels releases pro-inflammatory cytokines which attract macrophages to the site of lipid accumulation. Macrophages then become foam cells. The persistent signaling for macrophages and the build-up of foam cells associated with a chronic inflammatory response collectively lead to the development of atherosclerotic plaques (Moss & Ramji, 2016). Moreover, even slightly elevated levels of IL-1β, IL-6, and TNF-α seen in inflammation related to atherosclerosis are associated with a 10-25 percent increased risk for a non-fatal myocardial infarction or CVD death (Moss & Ramji, 2016; Golia et al., 2014). Chronic systemic inflammation also contributes to the pathogenesis of diabetes.

Diabetes affects approximately 30 million people in the U.S. and costs the U.S. healthcare system an estimated $245 billion each year (American Diabetes Association, 2018). Obesity, a primary risk factor for the development of insulin resistance and diabetes, is associated with elevated levels of circulating pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α (Calder et al., 2013). These cytokines are thought to damage insulin action cells, contributing to insulin resistance and ultimately to the development of type 2 diabetes (Lontchi-

Yimagou, Sobngwi, Matsha, & Kengne, 2013). Chronic inflammation is also involved in the development of chronic wounds because unremitting inflammation systemically and locally prevents wounds from progressing efficiently through the subsequent stages of healing (Gould et al., 2015).

Further, multiple studies have linked chronic low-grade inflammation to other age-related pathologies such as sarcopenia, osteoporosis, cognitive decline, neurodegenerative disease, frailty, arthritis, and certain cancers (Cevenini et al., 2013; Calder et al., 2017; Bettcher & Kramer, 2013; Minciullo et al., 2015; Ostan et al., 2015). In summary, many common chronic conditions in aging are linked to inflammaging and the associated high levels of pro-inflammatory cytokines in systemic circulation.

Inflammaging and Inflammatory Cytokines

Inflammaging is characterized by chronically high levels of pro-inflammatory cytokines in systemic circulation (Franceschi & Campisi, 2014; Calder et al., 2017). Cytokines are small signaling proteins that are synthesized by cells involved in the inflammatory response (e.g., neutrophils, macrophages) that recruit additional inflammatory cells to sites of inflammation and signal their activation or de-activation (Satish, 2015). Levels of pro-inflammatory cytokines in systemic circulation are known to increase with age and are involved in chronic disease pathologies. Moreover, pro-inflammatory cytokines in systemic circulation can cross the blood brain barrier and contribute to neuro-inflammation and cognitive decline (Banks, 2015). The pro-inflammatory cytokines IL-1β, IL-6, and TNF-α in particular have been linked to inflammatory conditions common in aging (Franceschi & Campisi, 2014; Calder et al., 2013).

IL-1β is primarily produced by leukocytes. It activates cells involved in the inflammatory response and promotes chronic inflammation by inducing pro-inflammatory cytokine gene

expression by cells involved in inflammation regulation (e.g., neutrophils) (Moss & Ramji, 2016). Moreover, IL-1β has been shown to be involved in smooth muscle cell proliferation, inflammatory cell recruitment, and synthesis of other inflammatory mediators such IL-6 and C-reactive protein (CRP) (Golia et al., 2014). IL-1β contributes to the development of CVD, insulin resistance, and even obesity (Christodoulidis et al., 2014; Guadarrama-López, Valdés-Ramos, & Martínez-Carrillo, 2014).

Rising IL-6 levels are also associated with aging and numerous chronic diseases (Franceschi & Campisi, 2014). According to Minciullo et al. (2015), with the absence of inflammation, serum levels of IL-6 are minute, but with aging and a dysregulation of the inflammatory responses, serum levels of IL-6 rise significantly. During the immune response, IL-6 is produced by cells in response to signaling by other pro-inflammatory cytokines such as IL-1β and TNF-α (Minciullo et al., 2015). IL-6 is involved in innate immune responses, hematopoiesis, and the initiation of inflammation (Minciullo et al., 2015; Christodoulidis et al., 2014). It also stimulates the liver to synthesize CRP, another pro-inflammatory mediator (Calder et al., 2013). IL-6 is associated with type-2 diabetes development, and in older adults it is associated with several chronic diseases, disability, and mortality (Lontchi-Yimagou et al., 2013; Xia et al., 2016). Further, low levels of IL-6 and other pro-inflammatory cytokines and high levels of anti-inflammatory cytokines have been found in centenarians, “suggesting that these extremely old persons do not suffer the ‘inflammaging’ noted in many older persons” (Calder et al., 2013).

TNF-α is another pro-inflammatory cytokine involved in many cellular processes that occur during the inflammatory response both systemically and locally (Xia et al., 2016). It has metabolic effects on bone, adipose tissue, and skeletal muscle leading to age-related cachexia

(Calder et al., 2013). TNF-α recruits leukocytes to sites of inflammation and promotes endothelial dysfunction and foam cell development in atherosclerosis (Christodoulidis et al., 2014). In obesity, TNF-α is over-synthesized by adipose tissue and is a risk factor for the development of insulin resistance (Guadarrama-López et al., 2014).

In summary, multiple age-related chronic inflammatory conditions are associated with increased levels of IL-1β, IL-6, and TNF-α in systemic circulation (Calder et al., 2013). Studies have shown that high levels of pro-inflammatory cytokines IL-6 and TNF-α in particular are related to increased risk of morbidity and mortality in older adults, increased risk of osteoporosis, increased risk of cerebrovascular and cardiovascular events, and an increased risk of cognitive decline (Minciullo et al., 2015; Michaud et al., 2013). Further, a sustained increase in levels of IL-1β, IL-6, and TNF-α has been noted in patients with chronic wounds, with levels of IL-1β increasing 400-fold (Satish, 2015).

Inflammaging and Chronic Venous Leg Ulcers

Chronic venous leg ulcers are the most common type of non-healing wound seen in aging adults. They affect about one percent of the American population, and ~3.6% of Americans over 65 years of age (Moor, Vachon, & Gould, 2009). Annual treatment-related costs are upwards of $35 billion in the U.S. alone (Nherera, Woodmansey, Trueman, & Gibbons, 2016). Moreover, people with CVLUs have a decreased quality of life because of the associated pain, restricted mobility, and social isolation (Finlayson et al., 2017).

Chronic venous leg ulcers develop as a result of venous insufficiency, venous hypertension and leaky vessels that collectively elicit an inflammatory response that fails to resolve (Chi & Raffetto, 2015). The chronic inflammation characteristic of CVLUs is marked by increased levels of pro-inflammatory cytokines both systemically (as seen with inflammaging)

and locally in the wound microenvironment (Chi & Raffetto, 2015; Gould et al., 2015; Makrantonaki et al., 2017). When the inflammatory process is dysregulated, wounds become "stuck" in the initial inflammatory stage of healing and subsequent healing stages are delayed or do not occur (Makrantonaki et al., 2017). The gold standard treatment for CVLUs is compression therapy, but despite this therapy, only 50-60 percent of CVLUs heal (Lazarus et al., 2013). As such, novel therapies are needed as adjuncts to compression therapy. The bioactive components of fish oil, EPA+DHA, have been shown to lower levels of pro-inflammatory cytokines and thus have anti-inflammatory effects systemically and locally (Calder, 2015). Thus EPA+DHA supplementation may be an effective oral adjunct intervention for CVLUs and potentially for other conditions linked to inflammaging.

Fish Oil: Potential Intervention for Reducing Effects of Inflammaging

The anti-inflammatory effects of fish oil were first noted in the Greenland Eskimo studies conducted by Bang, Dyerberg, & Nielsen (1971) who reported the link between a diet high in n-3 PUFAs from fish and the low incidence of ischemic heart disease as well as the absence of diabetes in this population. Since then, numerous studies have shown that the bioactive components of fish oil, EPA+DHA, can effectively decrease inflammation when consumed in appropriate amounts. Moreover, EPA+DHA supplementation has been reported to have no adverse effects in older adults (Villani et al., 2013). Therefore, EPA+DHA may be a promising therapy for older adults with conditions associated with inflammaging, such as CVLUs.

After consumption, EPA+DHA are incorporated into cell membrane phospholipids and reduce inflammation by blocking activation of nuclear factor κ B (NFκB), the key transcription factor for pro-inflammatory cytokines (Calder, 2015). Additionally, EPA+DHA are metabolized to lipid mediators (e.g., eicosanoids, resolvins) with anti-inflammatory and/or inflammation

resolving actions. However, typical Western diets are low in all n-3 PUFAs, but high in n-6 PUFAs (e.g., AA) that subsequently increase levels of pro-inflammatory cytokines (Figure 1). The recommended intake of n-6 and n-3 PUFAs is a ratio of 4:1, but the typical Western diet contains a ratio of ~15:1 (Wall et al., 2010). This is problematic because n-3 and n-6 PUFAs are metabolized competitively. This high ratio of n-6:n-3 PUFAs commonly seen in Western diets is believed to be a cause of inflammaging and thus a contributing factor in the development of many inflammatory diseases (Simopoulos, 2016). Reducing high n-6:n-3 ratios by increasing the intake of n-3 EPA+DHA may help improve health outcomes for the aging population.

Studies have shown that EPA+DHA supplementation can decrease plasma levels of pro-inflammatory cytokines in periods of acute inflammation. For example, McDaniel, Belury, Ahijevych, & Blakely (2008) reported that in acute wounds, EPA+DHA supplementation altered the production of pro-inflammatory cytokines. Not only has EPA+DHA been shown to reduce acute inflammation, it has also been shown to decrease levels of IL-1β, IL-6, and TNF-α systemically in patients with chronic inflammatory diseases. In patients with relapsing-remitting multiple sclerosis, a disease marked by increased inflammation, fish oil supplementation for 12 months significantly reduced serum levels of IL-1β, IL-6, and TNF-α (Ramirez-Ramirez et al., 2013). In another randomized controlled trial (RCT) of patients with chronic kidney disease, levels of plasma IL-1β were significantly lowered after 8 weeks of supplementation with fish oil (Deike et al., 2012). A systematic review of 23 articles of fish oil's effect on inflammation in patients with rheumatoid arthritis showed overall that n-3 PUFA supplementation had positive benefits in terms of symptom management including less joint swelling and pain, shorter duration of morning stiffness, and less use of non-steroidal anti-inflammatory drugs (Miles & Calder, 2012). Further, a meta-analysis of 68 RCTs concluded that EPA+DHA supplementation

significantly lowered levels of CRP, IL-6 and TNF-α in patients with chronic non-autoimmune disease and in healthy patients (Li, Huang, Zheng, Wu, & Li, 2014).

In older adults, some studies have shown that EPA+DHA therapy can decrease levels of pro-inflammatory cytokines during an acute inflammatory response. For example, a RCT that tested the effects of intravenous (IV) marine-derived n-3 PUFAs in older adults undergoing hip surgery found that it non-significantly decreased plasma IL-6 levels (Gopinath, Yelliboina, Singh, & Prasad, 2012). Likewise, a study by Barros et al. (2013) found that in elderly intensive care patients, levels of pro-inflammatory cytokines, including TNF-α, were reduced in patients who received an IV lipid emulsion containing EPA+DHA. The positive effects of EPA+DHA oral therapy have also been noted in healthy older adults. Kiecolt-Glaser et al. (2012) reported that EPA+DHA supplementation in sedentary, overweight, but healthy middle aged and older adults significantly decreased levels of IL-6 and TNF-α. In summary, multiple human studies have shown that EPA+DHA therapy reduces levels of pro-inflammatory cytokines systemically, but none have determined the effects exclusively in older adults with chronic inflammatory conditions.

Chapter III: Methodology

Study Design

This randomized, double-blind, placebo-controlled, repeated measures study examined adults in middle to late adulthood (N = 35; mean age = 61 years) with chronic venous leg ulcers (CVLUs) at Week 0 (baseline), and 4 and 8 weeks after dietary supplementation with EPA+DHA or placebo. Randomization of participants to one of the two treatment groups was performed by a person not associated with the study using a computer-generated randomization scheme. At Week 0, self-reported sociodemographic and comorbidity data were collected and body measurements

were recorded (height, weight) for body mass index (BMI) calculations. Fasting blood samples were collected at Weeks 0, 4 and 8 to quantify levels of fatty acids and pro-inflammatory cytokines. Participants were instructed to maintain their usual diets, but to exclude fish, seafood, algae, kelp and nutritional supplements until study completion. Participants received $250 after completing the study.

Study population

Participants were men and women 50 to 85 years of age diagnosed with a CVLU for ≥ 3 months, recruited over a period of 24 months from a large medical center's two out-patient wound clinics in the Midwest. The institutional review board (IRB) approved the study which was conducted at the university Clinical Research Center (CRC) associated with the medical center between August 2012 and July 2015 in compliance with ethical rules for human experimentation as stated in the 1975 Declaration of Helsinki. Participants were excluded from the study if they were allergic to fish or seafood, were prescribed warfarin therapy, had autoimmune conditions or any chronic inflammatory skin diseases (e.g. pyoderma gangrenosum), required non-steroidal anti-inflammatory drugs (NSAIDS) > 2x a week, or regularly used nutritional supplements (e.g., fish oil) or corticosteroids. All participants signed an informed consent document approved by the local IRB at the beginning of the study and the study protocol was registered December 12, 2012 at ClinicalTrials.gov (NCT01754506).

As a pilot study, 40 participants were recruited during the first 24 months of the study with an almost equal allocation to the two groups (n=21 for EPA+DHA and n=19 for Placebo). After accounting for attrition, the final sample included 35 people (16 for EPA+DHA and 19 for Placebo). This sample size provided 80% power to detect an effect size of 0.7 for within-group

comparisons and 80% power to detect an effect size of 0.9 for between-group comparisons at a two-sided significance level of 0.05.

EPA+DHA Supplement and Placebo

The softgel capsules (EPA+DHA or placebo) used in both groups were indistinguishable from each other. The manufacturer, J.R. Carlson Laboratories, Inc. (Arlington Height, IL), compounded the two types of softgels (EPA+DHA supplement and placebo) in a codified form. At Visit 1 (Week 0) EPA+DHA Group participants received instructions to take five opaque EPA+DHA softgels and one 81 mg tablet of acetylsalicylic acid (ASA) every day for the study interval. The five EPA+DHA softgels provided a total daily intake of approximately 1.5 g of EPA and 1.0 g of DHA (Table 1). This EPA/DHA ratio has been used in our previous work and was chosen because of evidence that EPA may have relatively stronger anti-inflammatory effects than DHA (Allam-Ndoul, 2016). The U.S. Food Drug Administration (FDA) evaluated the safety of EPA+DHA and concluded that a daily intake of EPA+DHA of up to 3.0 g/d is acceptable for the general public (FDA, 2009). Aspirin was administered because it has been found to enhance the inflammation-resolving action of the DHA-derived resolvin species. Placebo Group participants received identical-looking placebo softgels and 81mg ASA tablets and the same instructions for how to take the softgels as did the EPA+DHA Group participants. Five opaque placebo softgels provided a total daily intake of 5.2 g/d of light mineral oil, which is well below the therapeutic dose for constipation. Mineral oil is chemically inert and on ingestion the majority (98%) remains unabsorbed in the feces. The same daily mineral oil dose has been used as the placebo previous work with no adverse events being reported (McDaniel, Massey, & Nicolaou, 2011). Participants in both groups received the appropriate number of ASA tablets and softgels for 4 weeks of treatment at Visit 1. They were instructed to bring the empty bottles to Visit 2 (4 weeks later). At

Visit 2 another bottle of ASA and another bottle of softgels were administered for the subsequent 4 weeks of treatment. Participants were instructed to store the softgels in a refrigerator and to take five softgels and one ASA tablet daily with their evening meal to reduce the incidence of "fish burps" and to encourage compliance.

Plasma fatty acid measurements

Levels of fatty acids in plasma were quantified at the three study time points by the well-established gas chromatography/mass spectrometry (GC/MS) method. Lipids were extracted from plasma samples with 2:1 (v/v) chloroform:methanol and 0.24 ml 0.88% KCL (Parinandi, Weis, Natarajan, & Schmid, 1990). Fatty acid methyl esters were prepared using tetramethylguanidine at 100°C. Fatty acid methyl esters were analyzed by gas chromatography using a 30-m Omegawax™ 320 (Sigma-Aldrich, St. Louis, MO) capillary column. Oven temperature was started at 175°C and increased at a rate of 3°C/min until reaching 220°C. Flow rate of the carrier gas helium was 30 mL/min. Retention times were compared to standards for fatty acid methyl esters (Sigma-Aldrich, St. Louis, MO, & Matreya, LLC, Pleasant Gap, PA). The resultant values were expressed as % composition or ratios. This is routinely done to express fatty acid values and widely accepted in the field (Parinandi et al., 1990).

Plasma Pro-inflammatory cytokine measurements

Levels of IL-1β, IL-6 and TNF-α in plasma were quantified using Invitrogen (1600 Faraday Avenue Carlsbad, CA, 92008) Human ELISAs for IL-1β (cat# KHC0014), IL-6 (cat# KHC0061) and TNF-α (cat# KHC3011). Assay sensitivity and range for IL-6: <2 pg/mL and 7.8–500 pg/mL, respectively; for IL-1β: < 0.06 pg/mL and 0.31-20 pg/mL, respectively; for TNF-α: <1.7 pg/mL, standard curve range of 15.6–1,000 pg/mL. Aliquots of blood plasma were collected and stored at

-80^0 C until analyzed using the Biotek Powerwave plate reader (Highland Park, PO Box 998, Winooski, VT 05404) at the OSU, College of Nursing Stress Science Laboratory.

Statistical analyses

Descriptive statistics were used to summarize sample characteristics, stratified by treatment groups. Categorical variables are reported as frequencies and percentages, and continuous variables as mean and standard deviation (SD). The balance between EPA+DHA and Placebo Groups in baseline measures was tested using Chi-square statistics for categorical variables (e.g., sex) and two-sample t-tests for continuous variables (e.g., age and BMI). The effects of EPA+DHA on biomarkers (plasma PUFAs and cytokines) were estimated using mixed effect modeling to adjust for within-subject clustering from repeated measures. In each model, dependent variable was the level of the biomarker and the independent variables included treatment group (EPA+DHA or placebo), time (0, 4, 8 weeks), and their interaction. From the mixed effect modeling, we estimated the between-group comparisons at each time point and within-group comparisons of the levels of cytokines at 0, 4 and 8 weeks. If a significant baseline between-group difference was found, we adjusted for the baseline differences when conducting between-group comparisons at 4 and 8 weeks using mixed effect modeling. IBM's Statistical Package for the Social Sciences version 21.0 for Windows was used for the data analysis (SPSS, Chicago, IL). All tests were two-sided with significance level at $\alpha = .05$.

Chapter IV: Results

Participants' characteristics

Descriptive characteristics of the study participants are shown in Table 1. The two experimental groups of older adults were similar in age, sex, race, years of education, BMI, smoking history, wound characteristics and number of comorbidities.

Plasma levels of polyunsaturated fatty acid

Table 2 illustrates the means for select plasma PUFA levels by treatment group and study time points. The within-group analysis of the EPA+DHA Group showed that plasma PUFA levels were significantly higher for EPA at Week 4 ($p < .001$) and at Week 8 ($p < .001$) relative to Week 0. (Figure 1A) Similarly, DHA levels were significantly higher at Week 4 ($p < .001$) and Week 8 ($p < .001$) when compared to Week 0. (Figure 1B) Conversely, levels of AA were significantly lower at Week 4 ($p = .003$) and Week 8 ($p = .003$) than at baseline, as was the AA:EPA ratio (Week 4: $p < .001$; Week 8: $p < .001$) (Figure 1C), suggesting that the EPA study dose resulted in significantly increased proportions of EPA in the plasma that occurred partly at the expense of AA. Further, the n-6:n-3 ratio was significantly reduced over time in the EPA+DHA group (Week 4: $p < .001$; Week 8: $p < .001$). In comparison, there were no significant changes in the levels of EPA, DHA, AA, AA:EPA or n-6:n-3 across time in the Placebo Group.

As expected, the between-group comparisons showed that at Week 4 post enrollment, the EPA+DHA Group demonstrated significantly higher plasma levels of EPA ($p < .001$) and DHA ($p < .001$), lower levels of AA (marginally significant, $p = .055$), and significantly lower ratios of AA:EPA ($p < .001$) and n-6:n-3 ($p = .040$) than the Placebo Group. The same pattern was noted when comparing levels of EPA ($p < .001$), DHA ($p < .001$), AA ($p = .010$) and ratios of AA:EPA ($p < .001$) and n-6:n-3 ($p = .030$) between the two groups at Week 8 post enrollment. (Figure 1)

Plasma levels of cytokines IL-6, IL-1β and TNF-α

Figure 2 provides a summary of the results for plasma levels of IL-6, IL-1β and TNF-α by treatment group and study time points. The within-group analysis of the EPA+DHA Group showed that plasma IL-6 levels were significantly lower at Week 4 ($p = .008$) and at Week 8 ($p <$

.001) relative to Week 0. Similarly, IL-1β levels were significantly lower at Week 4 ($p < .001$) and Week 8 ($p < .001$) versus Week 0, and at Week 8 versus Week 4 ($p = .008$). Following this same pattern, levels of TNF-α were also lower at Week 4 ($p < .001$) and at Week 8 ($p < .001$) versus Week 0, and at Week 8 compared to Week 4 ($p = .005$). No significant within-group changes for levels of IL-6, IL-1β and TNF-α were detected in the Placebo Group over the study interval.

The between-group comparisons showed that levels of IL-6, IL-1β and TNF-α were significantly higher in the EPA+DHA Group at Week 0 compared to the Placebo Group ($p = .024$, $p < .001$ and $p < .001$, respectively). After adjusting for baseline difference, the treatment group had significant lower levels of IL-6 ($p = .008$), IL-1β ($p < .001$), and TNF-α ($p < .001$) at Week 4 compared to the placebo group. The treatment group also had significantly lower IL-6 ($p = .007$), IL-1β ($p < .001$), and TNF-α ($p < .001$) at Week 8 than the placebo group, after adjusting for the baseline differences.

Chapter V: Conclusion and Recommendations

Summary of Findings

The purpose of this randomized, double-blind controlled trial was to determine the effects of EPA+DHA oral therapy versus placebo therapy on levels of circulating pro-inflammatory cytokines in older adults with at least one known chronic inflammatory condition (CVLUs). We report that EPA+DHA therapy had a significant lowering effect on levels of IL-1β, TNF-α, and IL-6 after 4 weeks of therapy and an even greater lowering effect after 8 weeks of therapy. Specifically, for participants in the EPA+DHA Group, IL-6 decreased by 12% (0 - 4 weeks) and 22% (0 - 8 weeks), IL-1β decreased by 29% (0 - 4 weeks) and 44% (0 - 8 weeks), and TNF-α decreased by 12% (0 - 4 weeks) and 23% (0 - 8 weeks). There were no significant changes in

cytokine levels noted for participants in the Placebo Group over the study interval. After adjusting for baseline differences, the EPA+DHA group also had significantly lower levels of IL-1β, IL-6 and TNF-α at Weeks 4 and 8 compared to the Placebo Group.

Inflammaging, chronic low-grade inflammation seen with aging, results in high levels of circulating pro-inflammatory cytokines which contributes to the development of chronic disease and increases the risk for morbidity and mortality in older adults (Franceschi & Campisi, 2014). Decreasing these high levels of pro-inflammatory cytokines in the aging population may help to reduce the effects of or prevent chronic disease. We hypothesized that EPA+DHA oral therapy would be specifically effective in reducing levels of IL-1β, IL-6 and TNF-α in the participants of the present study due to their advancing age, chronic inflammatory condition (CVLUs), and Western diet, all known contributing factors to inflammaging (Franceschi & Campisi, 2014; Gould et al., 2015; Simopoulos, 2016). We report that 2.5 g/d of EPA+DHA oral therapy over 4 weeks and 8 weeks significantly reduced plasma levels of IL-1β, IL-6 and TNF-α. Our findings also show significantly reduced n-6:n-3 PUFA and AA:EPA ratios, suggesting that supplementation with EPA+DHA can increase plasma levels of EPA+DHA and reduce AA levels to combat the harmful inflammatory effects of a typical Western diet. Further, the dose of EPA+DHA used in the present study was within the FDA's safety recommendations and no adverse side effects were reported.

The current study findings of significantly lower levels of pro-inflammatory cytokines IL-6, IL-1β and TNF-α after 4 and 8 weeks of EPA+DHA therapy are consistent with some human studies reporting that EPA+DHA oral therapy reduces pro-inflammatory cytokine levels (McDaniel et al., 2008; Ramirez-Ramirez et al., 2013; Deike et al., 2012; Li et al., 2014; Gopinath et al., 2012; Barros et al., 2013; Kiecolt-Glaser et al., 2012). Conversely, our results

differ from Muldoon et al., (2016) who found that 1.4 g of EPA+DHA did not have a lowering effect on plasma levels of IL-6 and CRP in healthy adults. However, this is to be expected as the dose of EPA+DHA used in their study was considerably lower than the current study's dose (1.4g/d vs. 2.5 g/d), and previous studies report that a supplement dose of ≥2 g/d of EPA+DHA is needed to effectively decrease pro-inflammatory cytokine synthesis (Calder, 2015). Further, the participants in the current study had higher levels of pro-inflammatory cytokines at baseline that were likely due to their multiple chronic conditions compared with Muldoon et al.'s study wherein participants were healthy adults of various ages. The current study findings are in agreement with current literature reporting that people with high baseline levels of pro-inflammatory cytokines see a greater reduction in pro-inflammatory cytokine levels with EPA+DHA supplementation than people with low baseline levels (Li et al., 2014).

Conclusions

In the current study, EPA+DHA oral therapy had a significant lowering effect on plasma levels of select pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) associated with inflammaging after 4 weeks of therapy and an even greater lowering effect after 8 weeks of therapy. Moreover, EPA+DHA levels were significantly higher in the plasma at 4 weeks and 8 weeks compared to baseline, suggesting that participants consumed the supplement as instructed. Additionally, there were no reported side-effects associated with the intervention. Reducing chronically high levels of circulating pro-inflammatory cytokines may decrease systemic and local inflammation, and thus help prevent, delay, or reduce symptoms of many age-related inflammatory conditions such as CVLUs.

Limitations

Limitations of this study include the short duration of the study (8 weeks) and the small sample size (n=35) of participants from the Midwest. It is not known if the results of this study would be similar in larger samples of diverse adults from different regions of the U.S. who experience different environmental factors. Additionally, this study only tested the most common three pro-inflammatory cytokines associated with inflammation. It is not known whether EPA+DHA therapy lowers levels of other pro-inflammatory cytokines or increases levels of anti-inflammatory cytokines.

Implications

EPA+DHA supplementation is a promising low-risk, low-cost therapy for decreasing the significant negative clinical effects of inflammaging linked to high levels of circulating pro-inflammatory cytokines in aging adults. EPA+DHA plasma levels can be established within minutes via a finger stick. As such, clinicians can efficiently target people who are most likely to benefit from EPA+DHA therapy. Nurses, physicians, and other healthcare providers could promote EPA+DHA rich diets which may ultimately improve health outcomes in the aging population.

Recommendations

Additional studies are needed to test EPA+DHA oral therapy in larger, more diverse samples of middle-aged and older adults with and without chronic inflammatory conditions to garner more data and clarify mechanisms of action. However, given that the U.S. FDA has concluded that intake of EPA+DHA up to 3.0 g/d is acceptable for the public to safely use for anti-inflammatory purposes (FDA, 2009) and that the current study and previous studies have

reported no adverse effects associated with EPA+DHA therapy, aging adults may benefit from a dose between 2.0 g/d and 3.0 g/d to help prevent or reduce the harmful effects of inflammaging.

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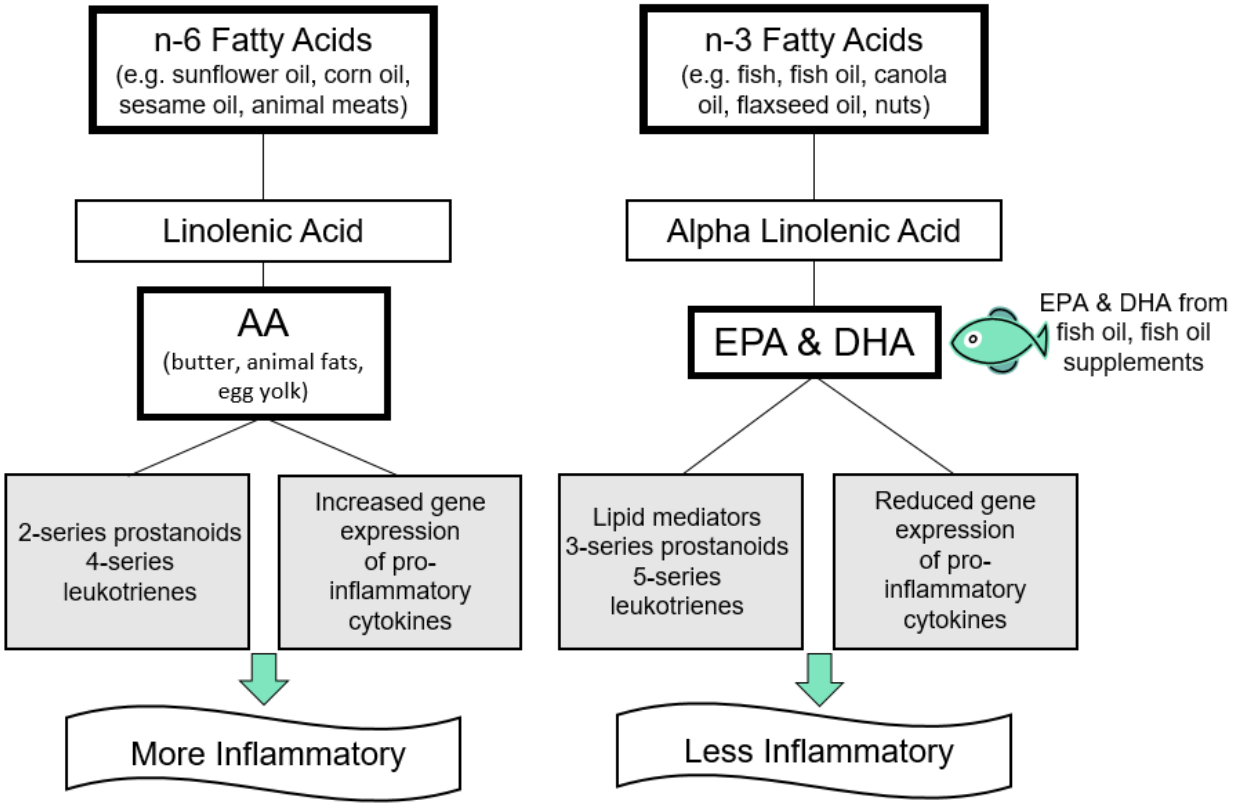
Figure 1. N-6 and n-3 polyunsaturated fatty acid metabolic pathways



AA= Arachidonic acid; DHA= Docosahexaenoic acid; EPA= Eicosapentaenoic acid

Table 1. Sociodemographic and Wound Characteristics of Participants

	EPA+DHA [a] (n = 16)	Placebo [a] (n = 19)
Age, mean years (SD)	60.3 (12.6)	60.9 (11.8)
Male (%)	10 (62.5)	11 (58)
Female (%)	6 (37.5)	8 (42)
White (%)	12 (75)	14 (75)
African American (%)	4 (25)	5 (26)
BMI, kilograms/meter^2 - mean (SD)	40.4 (8.2)	42.7 (13.8)
Wound Characteristics		
Size, baseline (cm^2) - mean (SD)	15.6 (34.4)	19.7 (23.2)
Estimated wound age		
< 6 months (%)	8 (50)	7 (36.8)
> 6 months (%)	8 (50)	12 (63.2)
Comorbidities		
Cardiovascular disease (%)	13 (81)	14 (74)
Diabetes (%)	8 (50)	7 (34)
Arthritis (rheumatoid, osteo) (%)	10 (63)	8 (42)
Depression (%)	2 (13)	6 (32)
Smoking history (%)		
Current smoker	5 (31)	2 (11)
Past smoker	7 (44)	8 (42)
Never	4 (25)	9 (47)

[a] No significant differences between groups

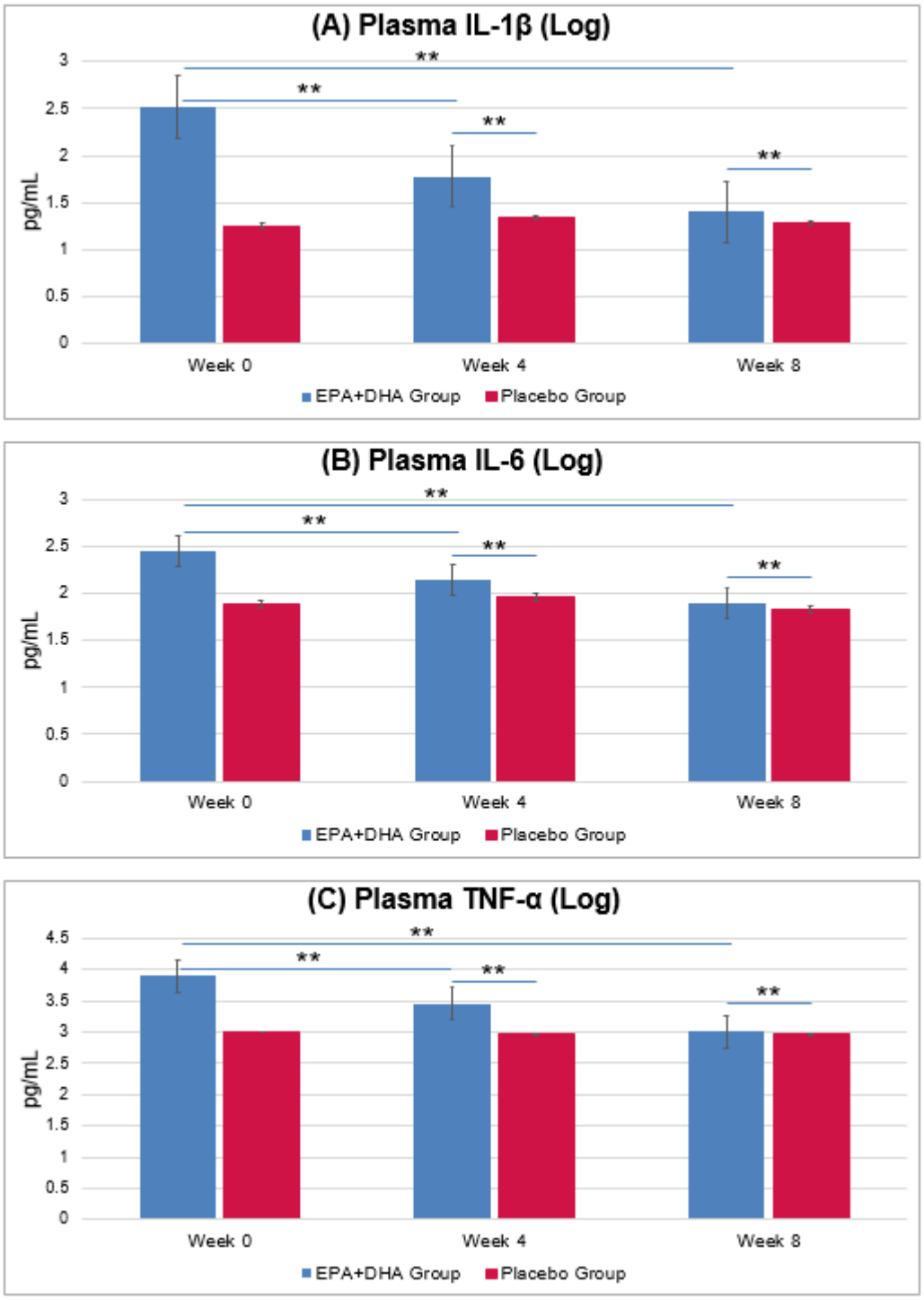
Table 2. Plasma Fatty Acid Measures for EPA+DHA Group and Placebo Group

Measures of Plasma Fatty Acid for EPA+DHA and Placebo Group Mean (SD)

Fatty Acids (µM/L)	Week 0		Week 4		Week 8	
	EPA+DHA	Placebo	EPA+DHA	Placebo	EPA+DHA	Placebo
EPA	0.52 (0.22)	0.52 (0.26)	2.21 (1.25)	0.46 (0.24)	2.33 (0.98)	0.48 (0.25)
DHA	1.47 (0.59)	1.50 (0.44)	2.77 (0.84)	1.46 (0.50)	3.09 (0.99)	1.49 (0.52)
AA	7.83 (2.45)	8.38 (2.00)	6.82 (1.83)	8.37 (2.49)	6.80 (2.16)	8.83 (2.61)
Total						
n-6	38.02 (4.32)	37.46 (3.81)	35.37 (4.78)	36.51 (4.68)	35.99 (5.77)	37.27 (4.10)
n-3	2.89 (0.55)	3.05 (0.71)	6.16 (1.83)	2.88 (0.50)	6.55 (1.61)	2.99 (0.67)
Ratios						
AA:EPA	13.75 (6.66)	16.28 (8.05)	4.19 (3.27)	15.39 (7.70)	3.46 (1.80)	15.81 (8.40)
n-6:n-3	15.21 (6.96)	12.69 (2.14)	8.35 (8.62)	12.90 (2.29)	8.04 (8.83)	12.91 (2.62)

EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid; AA= arachidonic acid
a = significantly different from baseline – within group ($p < .05$)
b = significantly different between groups @ Week 4 ($p < .05$)

Figure 2. Levels of pro-inflammatory cytokines (A) IL-1β, (B) IL-6, and (C) TNF-α at Baseline, Week 4, and Week 8 for EPA+DHA Group and Placebo Group



**Statistically significant